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ENZYMATIC COUPLED ASSAY PROCEDURES THAT EMPLOY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: THE SYNTHESIS OF OROTIDYLATE FROM RIBOSE

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SUMMARY

High-performance liquid chromatographic assay procedures have been designed to monitor the catalytic activities of ribokinase and phosphoribosyl α-1-pyrophosphate (PRibPP) synthetase. These methods are only of qualitative value, when crude protein extracts are to be examined, because of the presence of myokinase. However, the product of the PRibPP synthetase reaction, can be detected quantitatively even in crude protein extracts through the addition of two enzymes (orotate phosphoribosyltransferase and inorganic pyrophosphatase) that catalyze the conversion of PRibPP into a spectroscopically detectable nucleotide product (orotidylate).

INTRODUCTION

Ribose 5-phosphate (R5P), the compound that is produced from the hexose–monophosphate shunt in higher organisms, is the primary source of the ribose and deoxyribose portions of nucleic acids. In bacteria, however, R5P can also be produced directly from ribose though the use of ribokinase (RKase) activities[1]. Thereafter, R5P is directed toward the formation of nucleotides through its conversion to phosphoribosyl α-1-pyrophosphate (PRibPP). The enzyme that catalyzes this reaction is PRibPP synthetase (PRSase) which is present in higher animals[2] as well as in bacterial cells[3].

In our studies of the enzyme-catalyzed syntheses of orotidine monophosphate (OMP)[4,5], inosine monophosphate (IMP) or guanosine monophosphate (GMP)[6,7] and nicotinate mononucleotide (N_aMN)[8] we have made use of radioactively labeled PRibPP, and we and others have employed the RKase and PRSase activities of *Salmonella typhimurium* to accomplish the synthesis of several labeled PRibPP molecules[6,9–11]. In this paper we present the results of our attempts to design a high-performance liquid chromatographic (HPLC) procedure that would allow us to monitor the production of PRibPP from ribose in crude bacterial protein extracts and that would allow us to analyze kinetically a four-step (eqns. 1–4) synthesis of OMP. Such a procedure might serve as a general prototype for the kinetic analyses of coupled-enzymatic reactions.

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$$\text{Ribose} + \text{ATP} \overset{\text{RKase}}{\rightleftharpoons} \text{R5P} + \text{ADP} \quad (1)$$

$$\text{R5P} + \text{ATP} \overset{\text{PRSase}}{\rightleftharpoons} \text{PRibPP} + \text{AMP} \quad (2)$$

$$\text{PRibPP} + \text{orotate} \overset{\text{OPRTase}}{\rightleftharpoons} \text{OMP} + \text{PP}_i \quad (3)$$

$$\text{PP}_i + \text{H}_2\text{O} \overset{\text{PPase}}{\rightleftharpoons} 2\text{P}_i \quad (4)$$

MATERIALS AND METHODS

Materials

Ribose, R5P, orotate, adenosine triphosphate (ATP), PRibPP, and inorganic pyrophosphatase as well as EDTA and Trizma base were all purchased from Sigma (St. Louis, MO, U.S.A.). C-1[^{14}C]ribose was obtained from ICN whereas AG1-X8 ion-exchange resins were purchased from Bio-Rad. The Sephralyte quaternary amine ion-exchange HPLC column and guard-column resin were provided by Analytichem.

Enzyme preparations

RKase was isolated partially from *Salmonella typhimurium* cells by following the procedure for the purification of bacterial deoxyribokinase[1], whereas a PRSase preparation was obtained through the use of procedures described by Switzer[3]. Orotate phosphoribosyltransferase (OPRTase) was purified to near homogeneity from 10 lbs. of yeast by the following published procedures[4,12].

Radioactivity ribokinase assay procedure

This procedure is a modification of the method described by Schimmel *et al.*[1]. A 0.25-ml mini column of AG1-X8 was prepared in a Pasteur pipet. The acetate resin was converted to the chloride form by washing with 15 ml of 2 *M* hydrochloric acid followed by 15 ml of distilled water. The ribokinase assay mixture contained 10 m*M* ribose, 5 m*M* ATP, 10 m*M* $MgCl_2$, 1 m*M* EDTA, and 50 m*M* Tris buffer (pH 7.5). A volume of 0.025 ml of C-1[^{14}C]ribose was added to the assay mixture. In order to initiate the synthesis of R5P, a volume of 0.20 ml of the ribokinase preparation was added to 2 ml of the assay mixture and the incubation proceeded at room temperature. Aliquots (0.10 ml) were removed at 2, 5, 10, 60 and 285 min and the reaction in each aliquot was quenched by adding 2 ml of boiling water. Each sample was applied to the AG1-X8 column. The quenching vessel was then washed twice with 2 ml of water and this rinse was applied to the column. The column was then washed three times successively with 7 ml of water and the effluent was discarded. R5P was eluted from the column by washing with three 1 ml portions of 0.1 *M* hydrochloric acid. The total radioactivity in 0.1 ml of the assay mixture was 45,000 cpm and the total initial ribose concentration was calculated to be 10 m*M*. Under these conditions, then, an RKase activity of 125 nmoles R5P/h was determined by adding 0.2 ml of the enzyme preparation to a final volume of 2 ml. The rate of synthesis of R5P was linear over the 5-h incubation.

Spectroscopic PRibPP synthetase assay procedure

A coupled spectroscopic assay for PRSase has been designed which monitors the production of PRibPP by following the OPRTase-catalyzed PRibPP utilization to form OMP[12]. A volume of 0.2 ml of dilute PRSase was incubated with 2 ml of the assay solution consisting of 10 m*M* R5P, 10 m*M* $MgCl_2$, and 100 m*M* potassium phosphate (pH 8). Aliquots of 0.15 ml were removed at the times shown in Fig. 1 and filtered through a Millipore HA filter with 0.45-μm pore size. PRibPP concentrations were then determined by using the OPRTase assay and the PRibPP standard curve described below. An activity of 190 μmoles PRibPP formed per h was determined for PRSase under these conditions.

PRibPP concentrations were varied in an OPRTase assay mixture consisting of 150 μM orotate, 1 m*M* $MgCl_2$, and 50 m*M* potassium phosphate (pH 8). These concentrations were: 90 μM, 70 μM, 45 μM, 23 μM, 14 μM, 5 μM and 2 μM. A 0.015-ml volume of OPRTase was added to initiate the reaction and the resulting initial velocities were plotted *vs.* PRibPP concentration to construct a standard curve.

HPLC assay procedures for ribokinase and PRibPP synthetase

The HPLC equipment and general procedures employed in these studies have been described in detail elsewhere[13]. The Waters Associates Model 6000A pump, U6K injector and Model 440 UV detector (254 nm) were placed on-line with the Analytichem quaternary amine column. An isocratic elution buffer containing 0.10–0.15 *M* ammonium phosphate (pH 2.7) was first utilized to establish a relationship between the concentrations of injected ATP, ADP, and AMP and their elution peak heights. From this HPLC experiment linear standard curves were generated over ranges of 0–100 μM nucleotide concentrations. These data define, in addition to the absolute relationship between peak height and injected nucleotide concentration, the relative relationship between nucleotides. AMP and ADP concentrations can be compared to known ATP concentrations by dividing the AMP and ADP peak heights by 3.0 and 2.4 respectively.

(A) A 2-ml assay solution for PRibPP synthetase composed of 100 μM ATP, 100–1000 μM R5P, 1 m*M* $MgCl_2$ and either 50 m*M* triethanolamine or potassium phosphate buffer (pH 8), were incubated with 10–25 μl PRSase. Volumes of 0.3 ml were removed at known times over a 60-min period and filtered with 0.45-μm Millipore filters. Thereafter 0.02-ml fractions of those filtrates were injected onto the HPLC column. An alternative PRSase assay was sometimes employed where R5P was added last to initiate the reaction after the effect of PRSase on the ATP concentration in the absence of R5P had been monitored.

(B) The assay solution for ribokinase (0.9 ml) consisted of 100 μM ATP, 1 m*M* $MgCl_2$, 25 m*M* NaF (added to partially inhibit myokinase), and 50 m*M* triethanolamine (pH 7). This solution was brought to 37°C and then a volume of 0.01 ml RKase was added to the incubation mixture. Thereafter a 0.2-ml aliquot was removed after a 10-min time period to examine the effect of RKase on the ATP concentration in the absence of ribose. Finally ribose was added (100 μM) and aliquots were removed over a 60-min period and injected as described above.

(C) The assay solution for the four-step synthesis of OMP from ribose was composed of 1 m*M* ATP, 10 m*M* ribose, 10 m*M* $MgCl_2$, 25 m*M* NaF, 500 μM orotate, 50 m*M* triethanolamine and 50 m*M* potassium phosphate (pH 7). The syn-

thesis of OMP was initiated with the simultaneous additions of 0.005 ml RKase, 0.01 ml PRSase, 0.01 ml OPRTase, and 0.005 ml pyrophosphatase. Aliquots were removed successively over 90 min and injected as described above. Prior to these experiments the elution times of the UV-absorbing reactants were determined. These were 4.2, 4.5, 5.3, 5.9 and 7.3 min for AMP, orotate, ADP, OMP and ATP, respectively.

RESULTS AND DISCUSSION

Partially purified preparations of RKase and PRSase were obtained from *Salmonella typhimurium* and monitored using the radioactive and spectroscopic assay procedures described in the Materials and methods section. Both of these procedures proved to be time-consuming, and so we elected to see if HPLC assay procedures that characterize the utilization of ATP and the production of ADP and AMP could be employed to monitor RKase and PRSase activities in crude protein extracts. Such an assay would have to take into account the myokinase activity that is known to be present in these extracts[14]. In addition, we reasoned that an assay procedure that allowed us to characterize the synthesis of PRibPP from ribose via both the RKase and PRSase activities would be useful for the synthesis of labeled PRibPP from labeled ribose precursors.

In Fig. 1A are shown the reactant concentration changes for the HPLC assay system for ribokinase. As expected (Fig. 1A) the ATP, ADP and AMP concentrations change very little when ribose is not present in the incubation mixture although a net concentration of ADP is synthesized. Using the standard curves, this new ADP concentration is calculated to be less than 10 μM. In the presence of ribose, changes in the ATP, ADP and AMP concentrations all occur (Fig. 1A), and ADP appears to be synthesized initially via the RKase-catalyzed reaction and then utilized through

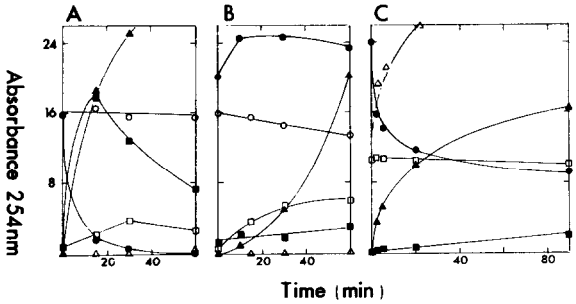

Fig. 1. Time-dependent changes in reactant concentrations (abosrbance at 254 nm) through incubation with ribokinase (RKase), PRibPP synthetase (PRSase) or these enzymes with orotate phosphoribosyltransferase (OPRTase) and inorganic pyrophosphatase (PPase). (A) RKase assay: ATP concentration in the presence (●) and absence (○) of ribose, ADP concentration in the presence (■) and absence (□) of ribose, AMP concentration in the presence (▲) and absence (△) of ribose. (B) RKase and PRSase assays in the presence of ribose 5-phosphate: ATP concentration during RKase incubation (○), ADP concentration during RKase incubation (□), AMP concentration during RKase incubation (△), ATP concentration during PRSase incubation (●), ADP concentration during PRSase incubation (■), AMP concentration during PRSase incubation (▲). (C) Assay components for RKase, PRSase, OPRTase and PPase: ATP (●), ADP (△), AMP (▲), orotate (□) and OMP (■). Assay and HPLC conditions were as described in the Materials and methods section.

the use of myokinase (eqn. 5). The known concentration of ATP (100 μM) is reduced to 10 μM after 15 min leading to the formation of 50 μM ADP and 40 μM AMP. After 90 min, no ATP and very little ADP remain whereas the AMP concentration is 70 μM. These results compare only qualitatively with those obtained from the radioactivity assay. Thus, this procedure will be useful in monitoring RKase activities, but only after the contaminating myokinase activity has been substantially reduced in the enzyme preparation under consideration.

$$2\ \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP} \tag{5}$$

The RKase assay has also been examined in the presence of R5P in place of ribose (Fig. 1B). This was completed in order to test whether the RKase preparation contained any PRSase activity. Although there was a small ATP concentration change (from 100 μM to 84 μM) over a 60-min time period, there occurred no net synthesis of AMP ($< 1\ \mu M$). Thus, the RKase preparation does not cotnain a PRSase activity. Once again however there occurred some synthesis of ADP (16 μM) after 60 min. Also shown in Fig. 1B is the HPLC assay for PRibPP synthetase. A lag period in AMP production was observed and approximately 55 μM AMP was produced after 60 min. Once again a small concentration of ADP (8 μM) was produced during this incubation. We believe therefore that this R5P-dependent AMP production reflects the PRSase activity in these preparations. The myokinase activity should not be a factor here since ADP would not be generated during this assay. We intend to obtain a homogeneous PRSase preparation and to perfect this assay procedure for this enzyme.

Because myokinase and perhaps other contaminating enzyme activities appeared to perturb significantly the RKase and PRSase HPLC assay systems, we have designed a more complex four-step coupled enzyme assay procedure which makes

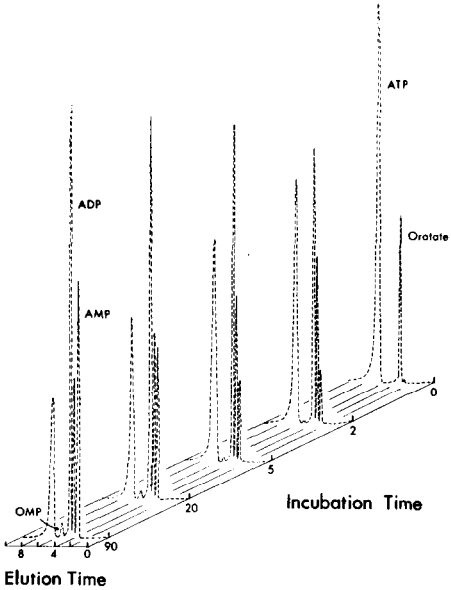

Fig. 2. HPLC elution profiles for the incubation of reactants with ribokinase, PRibPP synthetase, orotate phosphoribosyltransferase and inorganic pyrophosphatase. Elution and incubation times are in min.

use of the enzymes and reactants illustrated in eqns. 1–4. The time course for this assay procedure is illustrated in Fig. 1C, whereas the elution profiles of the various reactants over the incubation time are shown in Fig. 2. The appearance of OMP in this assay can only occur through the use of PRibPP which in turn must be synthesized from ATP and ribose via the RKase and PRSase activities. Thus, the allocation of ATP and ADP between the myokinase-catalyzed reaction and PRibPP synthesis can be monitored directly. Fig. 1C illustrates the assay in the presence of inorganic pyrophosphatase which alters the OPRTase-catalyzed reaction equilibrium (K_{eq} = 0.4, ref. 4) to favor OMP formation. The synthesis of OMP could not be detected during a similar incubation in the absence of the pyrophosphatase activity. As shown in Fig. 1C, ATP is rapidly utilized to form both AMP and ADP under these conditions, and a much slower rate of utilization of PRibPP is observed over the 90-min incubation as evidenced by the appearance of OMP. The actual concentration of reactants after the 90-min incubation period were calculated from standard curves to be 380 μM, 450 μM, 230 μM, 480 μM and 23 μM for ATP, ADP, AMP, orotate and OMP. We believe that this assay procedure will provide an effective method for detection of the synthesis of labeled OMP from ribose if the incubation is allowed to proceed for a longer period or if a more homogeneous preparation of RKase is provided. By placing the label (such as ^{14}C or ^{13}C-labeled positions of ribose) in the relatively stable OMP molecule rather than in PRibPP, the labeled material can be stored for long periods of time. Labeled PRibPP can then be regenerated from OMP at the appropriate time through an incubation with OPRTase and pyrophosphate.

In conclusion, a preliminary investigation of the feasibility of using HPLC assay procedures to monitor ribokinase and PRibPP synthetase has been completed. These procedures will be effective only after the myokinase activity has been removed from the enzyme preparations. However, for crude extracts which contain myokinase, a more complicated coupled HPLC assay procedure characterizes the rates of PRibPP formation. This procedure also will allow an examination of the synthesis of labeled PRibPP molecules.

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